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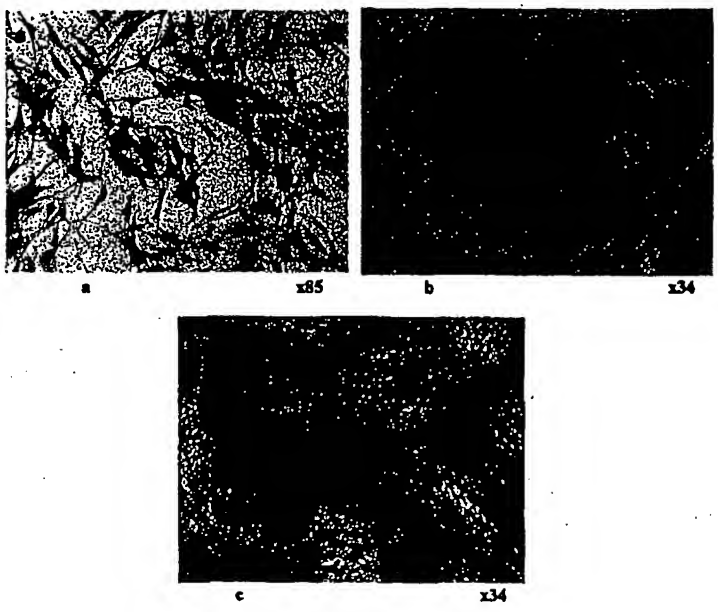


(57) Abstract

A multicellular *in vitro* assay for modelling the combined stage of angiogenesis namely the proliferation, migration and differentiation stages of cell development, wherein the assay comprises providing a dual culture of endothelial cells together with another cell–type e.g. fibroblast exhibiting interaction therewith to display the combined stages of angiogenesis *in vitro*.

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# A MULTICELLULAR *IN VITRO* ASSAY OF ANGIOGENESIS

This invention relates to an *in vitro* assay of angiogenesis and in particular a multicellular *in vitro* assay of angiogenesis.

Most populations of differentiated cells in vertebrates are subject to turnover through cell death and renewal. Some fully differentiated cells such as hepatocytes in the liver and endothelial cells lining the blood vessels simply divide to produce daughter cells of the same differentiated type. The proliferation rate of such cells is controlled to maintain the total number of cells. Thus if a large part of the liver is destroyed then the remaining hepatocytes increase their division rate in order to restore the loss.

Endothelial cells form a single cell layer that lines all blood vessels and regulates exchanges between the blood stream and the surrounding tissues. New blood vessels develop from the walls of existing small vessels by the outgrowth of these endothelial cells which have the capacity to form hollow capillary tubes even when isolated in culture. *In vivo*, damaged tissues and some tumours attract a blood supply by secreting factors that stimulate nearby endothelial cells to construct new capillary sprouts. Tumours that fail to attract a blood supply are severely limited in their growth.

The process where new vessels originate as capillaries which sprout from existing small vessels is called angiogenesis. It can therefore be seen that angiogenesis plays a major role in normal tissue development and repair and in the progression of some pathological conditions.

Once the vascular system is fully developed, endothelial cells of blood vessels normally remain quiescent with no new vessel formation. If disease or injury occurs the formation of new blood vessels can proceed normally, as in natural wound

healing, or be insufficient, as in chronic dermal ulcers, or there is deregulation of growth and an abnormal increase in vessel density ensues as in tumourogenesis, diabetic retinopathy, psoriasis and inflammation. Inhibition of inappropriate angiogenesis or enhancement of angiogenesis in non-healing wounds is therefore an extremely important target for drug discovery programmes. However, research in this area leading to new drug development has been hindered by the lack of *in vitro* models of angiogenesis.

Angiogenesis is an extremely complex process involving a wide range of growth factors, extracellular matrix molecules, enzymes and various cell types. Such a complexity of relationships has resulted in major difficulties in developing an *in vitro* assay which models the entire *in vivo* process. Angiogenesis can be subdivided into three phases: proliferation, migration and differentiation. Assays exist which model each of these phases separately. Simple *in vitro* tests measure changes in proliferation of a range of cell types and assess migration over basement membrane proteins. Current *in vitro* assay systems which depend on provision of a protein matrix effectively measure the ability of endothelial cells to differentiate.

Assay systems measuring differentiation involve the formation of cord like structures by endothelial cells. All such systems depend on supplying the cells with exogenous basement membrane proteins on which the cells migrate to form tubules. Cell migration occurs over relatively short time periods of 2-16 hours to give a three dimensional structure. In addition to the proteins, many of the systems require the provision of growth factors to produce acceptable tubule formation. The time scale over which tubules are formed provides an excellent test for inhibition of differentiation but is not so useful when testing for enhancement.

The assay systems described above come closest to modelling angiogenesis but none of them combine all three of the stages required for angiogenesis.

The object of the present invention is to obviate or mitigate the aforesaid disadvantages by providing an *in vitro* assay of angiogenesis which is dependent on all three stages of angiogenesis and can be used to examine both stimulation and inhibition of angiogenesis.

According to one aspect of the present invention there is provided a multicellular *in vitro* assay for modelling the combined stages of angiogenesis namely the proliferation, migration and differentiation stages of cell development, wherein the assay comprises providing a dual culture of endothelial cells together with another cell-type exhibiting interaction therewith to display the combined stages of angiogenesis *in vitro*.

According to an aspect of the invention such an assay relies on use of a dual culture of fibroblasts and endothelial cells and requiring no additional growth factors other than in standard culture medium. It is postulated that the interaction of these cell types is dependent on cell signalling mechanisms therebetween. The non-reliance on additional growth factors is remarkable and unanticipated considering past research on the subject.

According to another aspect of the present invention there is provided a method of screening agents for promoting or inhibiting angiogenesis comprising cultivating a co-culture of endothelial cells together with another cell-type (preferably interstitial cells such as fibroblasts), exhibiting interaction therewith to display the combined stages of angiogenesis, providing a plurality of test containers for same and presenting said agent in controlled amounts to said cultures and observing the containers to monitor angiogenesis. The screening method may be readily automated and angiogenesis may

be monitored by known automated counting techniques, image analysis or by spectrographic methods.

Preferably the assay comprises the steps of

(a) setting up growth containers suitable for sustaining dual cell cultures and having a suitable culture medium for sustaining at least growth of endothelial cells therein

(b) seeding a dual culture of human fibroblasts and human endothelial cells to obtain a pre-determined target ratio thereof

(c) incubating same without the provision of any exogenous growth factors

(d) monitoring the progress of the cells from an initial proliferation phase until a confluent monolayer is produced.

(e) changing the culture medium at regular intervals throughout the proliferation, migration and differentiation stages of the cell development.

Preferably the fibroblasts are Human Adult Dermal Fibroblasts and the endothelial cells are Human Umbilical Vein Endothelial Cells (HUVEC).

The cell ratio in the dual culture of Human Adult Dermal Fibroblasts to Human Umbilical Vein Endothelial Cells (HUVEC) is preferably from about 2:1 to 8:1.

Advantageously the culture medium of the dual culture is changed every 48 hours.

According to another aspect of the invention there is provided an assay kit including a vessel provided with culture medium appropriate for sustaining fibroblasts and endothelial cells, and seeded with said cells in a predetermined ratio as a

dual culture, wherein the cells are preferably Human Adult Dermal Fibroblasts and Human Umbilical Vein Endothelial Cells (HUVEC) respectively, the viability of the cells in said vessel being monitored for about 24 hours before sending to customers.

Preferably the vessel contains a cell ratio of Human Adult Dermal Fibroblasts to Human Umbilical Vein Endothelial Cells (HUVEC) of about 2:1 to 8:1.

A preferred test kit for use in a multicellular *in vitro* assay comprises a culture vessel seeded with a dual culture of Human Adult Dermal Fibroblasts and Human Umbilical Vein Endothelial Cells (HUVEC) having a cell ratio of about 2:1 to 8:1, said kit further comprising, growth medium capable of sustaining Endothelial cell growth, fixative, blocking buffer, washing buffer, reagents and antibodies for suitable visualisation.

Preferably the reagents for visualisation are those for use in von Willebrand Immunoassay or PECAM-1 Immunoassay.

According to a further aspect of the present invention there is provided a multicellular *in vitro* assay comprising a dual culture of endothelial cells and fibroblasts, preferably Human Adult Dermal Fibroblasts and Human Umbilical Vein Endothelial Cells (HUVEC) and being sustainable in a culture medium, said culture medium capable of sustaining at least endothelial cell growth, the dual culture having been seeded with a cell ratio of about 2:1 to 8:1 of Human Adult Dermal Fibroblasts to Human Umbilical Vein Endothelial Cells (HUVEC) wherein the assay is used to model *in vivo* angiogenesis for use particularly in the likes of drug research or tumour therapy whereby an inhibition of the angiogenesis model by a test drug would indicate its suitability for use in tumour therapy. An enhancement of the angiogenesis model by a test drug would indicate its suitability for use as a wound healing agent.

By virtue of this invention there is provided a multicellular *in vitro* assay which enables examination and modelling for each stage of angiogenesis namely each of the proliferation, migration and differentiation stages of cell development.

The invention will now be described by way of reference to the figures below and also by way of the following examples.

**Figure 1a-c:** show the development of the tubules over a period of 1,7 and 14 days

**Figure 1a:** Day 1. The darkly stained HUVEC (brown) are clearly visible, positioned on the surface of the fibroblast (blue) monolayer. (x85).

**Figure 1b:** Day 7. Thread-like tubules are forming in the confluent fibroblast monolayer. (x34).

**Figure 1c:** Day 14. An intricate network of thickened, anatomising vessels has formed, many originating from areas with high fibroblast concentration. (x34)

**Figure 2a:** A marked increase in tubule formation on addition of human recombinant vascular endothelial growth factor (VEGF), $10ngml^{-1}$. (x34).

**Figure 2b:** A marked decrease in tubule formation on addition of anti-human recombinant VEGF neutralising antibody, $10\mu gml^{-1}$ in combination with human recombinant VEGF $10ngml^{-1}$ (x34).

**Figure 3a:** Incubation with U-87-MG conditioned medium leads not only to the inhibition of tubule

formation but also to a massive increase in the number of HUVEC. Note some tubules form at the edges of the large HUVEC "islands". (x34).

**Figure 3b:** Incubation with GO-G-CCM conditioned medium effects a marked reduction in HUVEC proliferation. Those cells present, however, have formed small lengths of tubules. (x34)

**Figure 4:** Shows an image of tubule formation at day 14, developed by the PECAM-1 immunoassay using BCIP/NBT substrate (alkaline phosphatase compatible). (x34)

**Figure 5:** Shows an image of Collagen IV expression in the tubules at day 14. (x85)

**Figure 6:** A photographic image of a vertical cross section of the cell layer in the tubule assay at day 14 as seen through the electron microscope (original magnification x7,500).

The cultures of Figures 2a and 2b were incubated for 14 days and are therefore directly comparable to Figure 1c.

Visualisation in Figures 1-3 are by von Willebrand factor immunoassay, using DAB substrate, and haematoxylin counterstain.

In accordance with the present invention there is provided an *in vitro* assay for angiogenesis dependent on appropriate cell signalling mechanisms using a dual culture and requiring no additional growth factors. Both stimulation and inhibition of angiogenesis can be demonstrated using this technique.

Furthermore the assay system of the present invention combines all three stages of angiogenesis namely proliferation, migration and differentiation.

The assay system involves co-culture of Human Umbilical Vein Endothelial Cells (HUVEC) with Human Adult Dermal Fibroblasts. Under the conditions provided the cells form a series of anastomosed tubules.

The Human Umbilical Vein Endothelial Cells (HUVEC) are commercially available from suitable outlets and in this case are bought in cryopreserved form. Prior to employment in the tubule assay, the cells are routinely passed and cultured in any suitable commercially available Endothelial Growth Medium, EGM, containing 2% foetal calf serum. The HUVEC are used at passes 2 to 6 in the assay.

The Human Adult Dermal Fibroblasts are cultured in house from skin samples obtained from the local hospitals. Prior to employment in the tubule assay, the cells are routinely passed and cultured in Dulbecco's Modified Eagle's Medium plus 10% foetal calf serum. The fibroblasts are used at passes 6 to 10 in the assay.

The tissue culture treated vessels to be used in the assay are equilibrated by pre-incubation with EGM, plus and minus treatments, for a period of 30 minutes at 37°C, 5% $CO_2$ humidified atmosphere. Although 12-well and 24-well tissue culture treated plates are normally used others may equally well be employed and the volumes added are 1ml per well for 12-well plates and 0.5ml per well for 24-well plates. The cells are harvested using any suitable commercially available Trypsin solution for fibroblasts and any suitable commercially available Trypsin-EDTA solution for HUVEC. The cells are resuspended in EGM and counted. Immediately before use, the cells are expressed through a syringe and needle (23G x 1¼) to ensure good dispersal.

The two types of cell are thoroughly mixed at the required densities and seeding ratio (which can be between 2:1 and 8:1, fibroblasts to HUVEC) and added to the plates. In order to ensure an even distribution over the growth surface, the plates are gently agitated in a random fashion. This prevents a pooling of cells in the centre of the wells.

Cell ratios and seeding densities are of paramount importance in this assay. These can vary with each HUVEC line employed and must be established whenever a new line is introduced, to maximise conditions for tubule formation.

The co-cultures are normally incubated over a period of 14 days with complete medium changes approximately every two days. Rudimentary tubule development is evident from around day 4, but, as with all cell types, variations can occur and tubules may form earlier. The whole process can be accelerated to a seven day period or less by increasing the seeding densities whilst maintaining the established ratio. This, however, is not always desirable as the effects of any treatments may be better seen over the long term rather than the short term.

To monitor the progress of the assay, four time points are normally used over a 14 day growth period. This may be altered to suit requirements. At each time point the medium is discarded from the growth vessel and the cells fixed in cold (-20°C) 70% Ethanol for 30 minutes at room temperature. At this point the plate may be washed and stored in phosphate buffered saline at 4°C until completion of the experiment when all the cultures are developed at the same time, or each time point may be processed separately.

To date visualisation of the tubules involves the targeting of one of two cell markers in the HUVEC line by immunoassay. These markers are the glycoprotein von Willebrand factor and the cell adhesion molecule PECAM-1.

Human von Willebrand factor (factor VIII R:Ag) is a multimeric plasma glycoprotein. It mediates platelet adhesion to vessel walls and serves as a carrier and stabiliser for coagulation factor VIII. The factor is synthesised constitutively by endothelial cells. Platelet Endothelial Cellular Adhesion Molecule or PECAM-1 is a 130-KD integral membrane glycoprotein that is the member of the Ig super family and is found constitutively on the surface of endothelial cells, particularly at intercellular junctions. It is also expressed on the surface of platelets and leukocytes.

The immunoassay process involves a two step indirect method where an enzyme-conjugated secondary antibody reacts with an unlabelled primary antibody bound to the cell marker. A substrate solution is added and this reacts with the enzyme complex to produce an insoluble coloured end product. In this way the endothelial tubules are visualised. The co-cultures may be counterstained with haematoxylin nuclear stain. This aids visualisation of the fibroblast monolayer.

Quantitative assessment of the tubules may be achieved by a variety of methods, ranging from manual counting to video imaging and computerised image analysis.

When the HUVEC cells and fibroblasts are incubated together in co-culture without the addition of any exogenous growth factors, but with the complete replacement of the culture medium every two days, the cells initially pass through a proliferative stage which continues until a confluent monolayer is produced. At day 1 the culture consists of a background of fibroblasts with small islands of endothelial cells (Figure 1a). The endothelial cells, continuing to proliferate, enter a migratory phase where they can be seen to move within the fibroblast layer to form thread-like tubular structures at approximately day 7 (Figure 1b). These structures eventually extend and join up to form an intricate network resembling the capillary bed of the chick chorioallantoic membrane at about day 14 (Figure 1c). The

"vessels" formed by this process can often be seen to originate from the islands of HUVEC formed during the proliferative phase. High concentrations of fibroblasts are nearly always visible in the area from which the HUVEC have migrated. By day 14 the tubules are wider and thicker with patent lumina which can be visualised with phase-contrast microscopy.

Both the seeding density of the two cell types and the ratio of HUVEC to fibroblasts are extremely critical. The rate at which the HUVEC divide also appears to be critical. By using HUVEC which have widely differing doubling times it can be seen that when the doubling time is short, and therefore the HUVEC are growing very quickly, the outcome tends to be large islands of undifferentiated HUVEC. This would tend to indicate that there is a critical point during the process when intracellular signals between fibroblast and HUVEC initiate the differentiation process.

Experiments were also carried out to show the possibility of using the assay to show the inhibition or stimulation of angiogenesis by a sample under test for example for testing the effects of new drugs.

Vascular endothelial growth factor (VEGF) is a recognised mitogen of endothelial cells and stimulates angiogenesis. Manipulation of the *in vitro* system was confirmed by adding human recombinant VEGF at the start of the experiment and at each medium change. As a result, tubule formation was much enhanced with networks of numerous arcades (Figure 2a).

Conversely, when anti-human recombinant VEGF neutralising antibody was added with VEGF, tubule formation was markedly reduced (Figure 2b). The system was also tested using conditioned medium (serum free) from various tumour cell lines including that from U-87-MG (human glioblastoma cell line) which was available from the local hospital and GO-G-CCM (human brain astrocytoma) available from the European Collection Of Animal Cell Cultures (ECACC), for it has been hypothesised that

tumour cells can control angiogenesis and in turn favour growth. U-87-MG caused a massive proliferation of HUVEC with very little tubule formation (Figure 3a) whereas GO-G-CCM much reduced HUVEC proliferation and tubule formation (Figure 3b).

These results demonstrate the flexibility of the assay and the response to materials which have different modes of action.

In this way the assay can be used to screen for inhibitors and enhancers of angiogenesis.

In Figure 5 there is shown an image of Collagen IV expression in the tubules at day 14. *In vivo,* Extra Cellular Matrix (ECM) proteins are laid down by the developing capillaries of neovasculature and this *in vitro* image shows that Collagen IV is selectively expressed by the endothelial cells. This is further evidence that the assay is indeed mimicking the in vivo development of vessels.

Figure 6 shows a photographic image of a vertical cross-section of the cell layer in the tubule assay (day 14) as seen through an electron microscope (original magnification x 7,500). This quite clearly shows a tubule composed of several endothelial cells (shown by arrowheads) encompassing a central lumen (shown by arrow). This presents further proof that the assay is in fact producing tubules with a central "cavity" or lumen.

A control study was also set up to show that not all cells would function if substituted for those currently used in the assay of the present invention. The control study showed HUVEC co-cultured with human umbilical artery smooth muscle cells (HUASMC), cells which form a close association with the endothelial cells *in vivo*. No tubules formed.

It is envisaged that the above invention will give a more accurate study of *in vivo* angiogenesis by using the *in vitro* model for experiments to see the effect of various external

factors on same. For example, studying the effect of particular drugs (particularly in the field of tumour therapy) will be greatly assisted by this invention. The assay can be used to determine whether the particular drug under test would inhibit angiogenesis thereby inhibiting tumour growth or whether it would enhance angiogenesis it thereby having applications in wound healing therapy. *In vivo*, damaged tissues and some tumours attract a blood supply by secreting factors that stimulate nearby endothelial cells to construct new capillary sprouts. It can therefore be shown by using this assay whether a particular test drug can prevent the stimulation of the endothelial cells thereby preventing the tumours from attracting a blood supply. Tumours that fail to attract a blood supply are severely limited in their growth. The present invention is also extremely valuable in the study of angiogenesis *per se*.

It is also envisaged that culture vessels will be seeded with viable co-cultures which will be grown up at the preferable cell ratio of between about 2:1 and 8:1, fibroblasts to HUVEC. After approximately 24 hours of co culture the vials would then be suitably packaged in the form of a kit. The kit will also contain the necessary ingredients required to keep the co-culture viable, and also for visualisation of results ( by means of von Willebrand Immunoassay or PECAM-1 Immunoassay).

The preferred test kit for use in the multicellular in vitro assay has a culture vessel seeded with a dual culture of Human Adult Dermal Fibroblasts and Human Umbilical Vein Endothelial Cells (HUVEC) having a cell ratio of about 2:1 to 8:1, a quantity of growth medium capable of sustaining Endothelial cell growth, fixative, blocking buffer, washing buffer, and the reagents and antibodies for suitable visualisation by von Willebrand Immunoassay or PECAM-1 Immunoassay.

Therefore the kit will also contain the following components for use in a von Willebrand Immunoassay:

(i) a primary antibody - rabbit anti-human von Willebrand Factor;

(ii) a secondary antibody - goat anti-rabbit IgG (whole molecule) Horse radish Peroxidase conjugate; and

(iii) a substrate - Horse radish Peroxidase substrate with insoluble end product

and the following components for use in a PECAM-1 Immunoassay:

(i) a primary antibody - mouse anti-human PECAM-1;

(ii) a secondary antibody - goat anti-rabbit IgG (whole molecule) Alkaline Phosphatase conjugate; and

(iii) a substrate - Alkaline Phosphatase substrate with insoluble end product.

The completed kits will then be sent out to customers for use in their own research such as angiogenesis research, drug study groups and/or research into wound repair.

**Claims**

1. A multicellular *in vitro* assay for modelling the combined stages of angiogenesis namely the proliferation, migration and differentiation stages of cell development, wherein the assay comprises providing a dual culture of endothelial cells together with another cell-type exhibiting interaction therewith to display the combined stages of angiogenesis *in vitro*.

2. A multicellular *in vitro* assay for modelling the combined stages of angiogenesis according to claim 1 wherein the dual culture comprises a mixture of interstitial cells, endothelial cells and standard culture medium with no additional growth factors.

3. A multicellular *in vitro* assay for modelling the combined stages of angiogenesis particularly for screening agents for promoting or inhibiting angiogenesis comprising cultivating a co-culture of endothelial cells together with another cell-type exhibiting interaction therewith to display the combined stages of angiogenesis, providing a plurality of test containers for same and presenting said agent in controlled amounts to said cultures and observing the containers to monitor angiogenesis.

4. A multicellular *in vitro* assay for modelling the combined stages of angiogenesis according to claim 3 wherein the screening method is automated and angiogenesis is monitored by known automated counting techniques.

5. A multicellular *in vitro* assay for modelling the combined stages of angiogenesis according to claim 3 wherein the angiogenesis is monitored by image analysis.

6. A multicellular *in vitro* assay for modelling the combined stages of angiogenesis according to claim 3 wherein the angiogenesis is monitored by spectrographic methods.

7. A multicellular *in vitro* assay for modelling the combined stages of angiogenesis according to claim 3 wherein the other cell type is interstitial cells.

8. A multicellular *in vitro* assay for modelling the combined stages of angiogenesis according to claims 2 or 7 wherein the interstitial cells are fibroblasts.

9. A multicellular *in vitro* assay for modelling the combined stages of angiogenesis wherein the method comprises the steps of

(a) setting up growth containers suitable for sustaining dual cell cultures and having a suitable culture medium for sustaining at least growth of endothelial cells therein

(b) seeding a dual culture of human fibroblasts and human endothelial cells to obtain a pre-determined target ratio thereof

(c) incubating same without the provision of any exogenous growth factors

(d) monitoring the progress of the cells from an initial proliferation phase until a confluent monolayer is produced.

(e) changing the culture medium at regular intervals throughout the proliferation, migration and differentiation stages of the cell development.

10. A multicellular *in vitro* assay for modelling the combined stages of angiogenesis according to claims 8 or 9 wherein the fibroblasts are Human Adult Dermal Fibroblasts and the endothelial cells are Human Umbilical Vein Endothelial Cells (HUVEC).

11. A multicellular *in vitro* assay for modelling the combined stages of angiogenesis according to claim 10 wherein the cell ratio in the dual culture of Human Adult Dermal Fibroblasts to Human Umbilical Vein Endothelial Cells (HUVEC) is from about 2:1 to 8:1.

12. A multicellular *in vitro* assay for modelling the combined stages of angiogenesis according to claims 3 or 9 wherein the culture medium of the dual culture is changed every 48 hours.

13. A kit for use in a multicellular *in vitro* assay for modelling the combined stages of angiogenesis wherein the kit comprises a vessel provided with culture medium appropriate for sustaining fibroblasts and endothelial cells, and seeded with said cells in a predetermined ratio as a dual culture, wherein the cells are preferably Human Adult Dermal Fibroblasts and Human Umbilical Vein Endothelial Cells (HUVEC) respectively.

14. A kit according to claim 13 wherein the vessel contains a dual culture having a cell ratio of Human Adult Dermal Fibroblasts to Human Umbilical Vein Endothelial Cells (HUVEC) of about 2:1 to 8:1.

15. A kit according to claim 14 wherein the kit comprises a culture vessel seeded with the dual culture, said kit further comprising, growth medium capable of sustaining Endothelial cell growth, fixative, blocking buffer, washing buffer, reagents and antibodies for visualisation.

16. A kit according to claim 15 wherein the reagents for visualisation are those for use in a von Willebrand Immunoassay.

17. A kit according to claim 15 wherein the reagents for visualisation are those for use in a PECAM-1 Immunoassay.

18. A multicellular *in vitro* assay, suitable for modelling *in vivo* angiogenesis, for use in therapeutic evaluation of the

potential indication of a drug comprising the provision of a dual culture of endothelial cells and interstitial cells in a culture medium, said dual culture being viable and sustainable in said culture medium for a period sufficient to complete the assay, introducing the drug to be evaluated to the dual culture, and observing the effects thereof on cell behaviour, particularly with regard to angiogenesis, whereby an inhibition of angiogenesis by said drug indicates a potential for use in tumour therapy and an enhancement of the angiogenesis model by said drug indicates a potential use as a wound healing agent.

**19.** A multicellular *in vitro* assay, suitable for modelling *in vivo* angiogenesis, for use in therapeutic evaluation of the potential indication of a drug according to claim 18 wherein the interstitial cells are fibroblasts.

**20.** A multicellular *in vitro* assay, suitable for modelling *in vivo* angiogenesis, for use in therapeutic evaluation of the potential indication of a drug according to claim 19 wherein the fibroblasts are Human Adult Dermal Fibroblasts and the endothelial cells are Human Umbilical Vein Endothelial cells (HUVEC).

**21.** A multicellular *in vitro* assay, suitable for modelling *in vivo* angiogenesis, for use in therapeutic evaluation of the potential indication of a drug according to claims 18-20 wherein the dual culture provided has been seeded with a cell ratio of about 2:1 to 8:1 of Human Adult Dermal Fibroblasts to Human Umbilical Vein Endothelial Cells (HUVEC)

# ANGIOGENESIS ASSAY

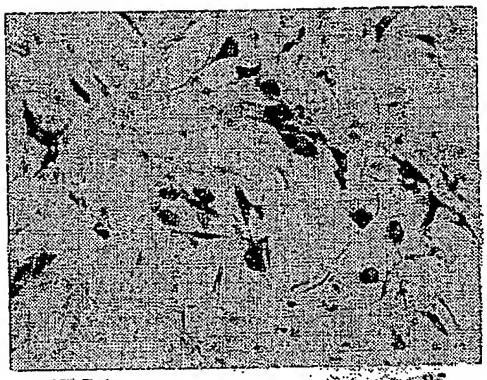

FIG 1a x85

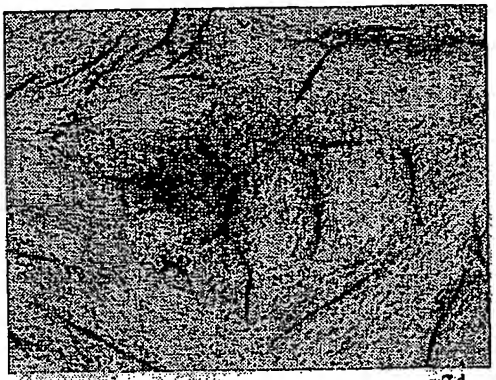

FIG 1b x34

FIG 1c x34

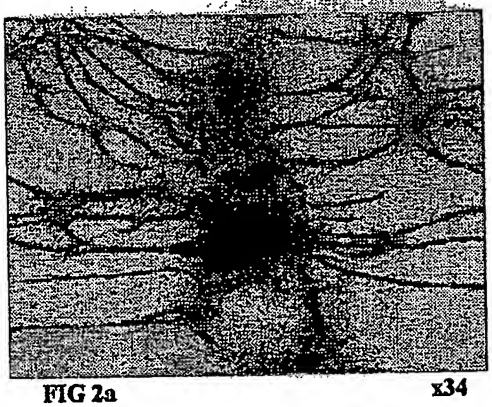

FIG 2a x34

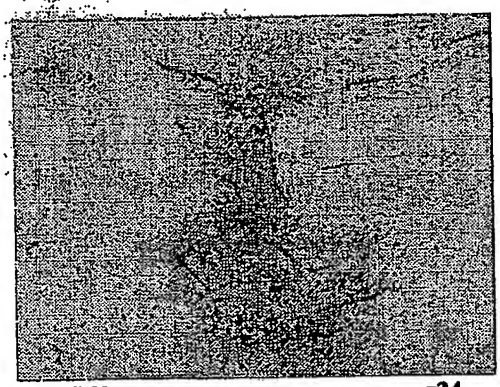

FIG 2b x34

# ANGIOGENESIS ASSAY

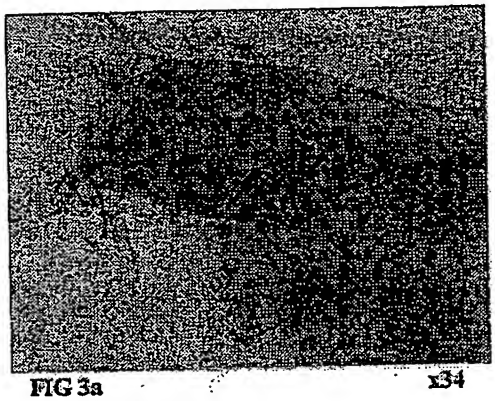

FIG 3a x34

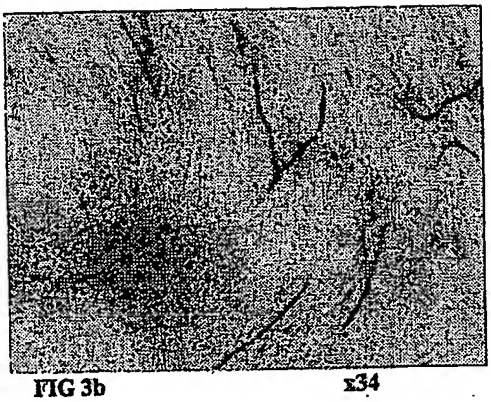

FIG 3b x34

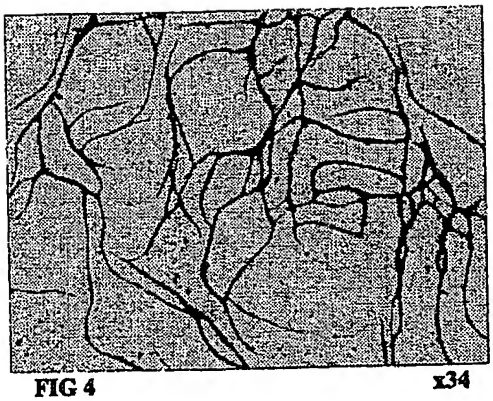

FIG 4 x34

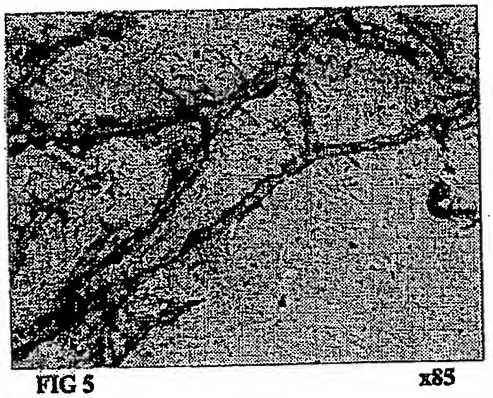

FIG 5 x85

# ANGIOGENESIS ASSAY

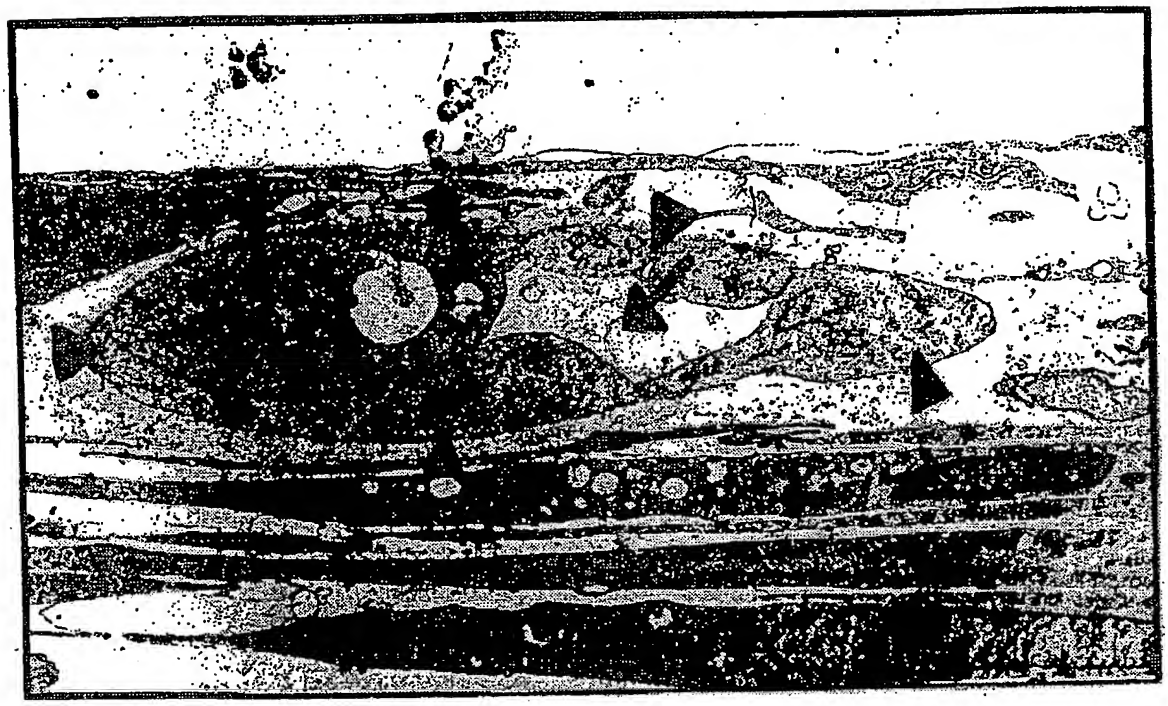

FIG 6 (original magnification) x7,500

# INTERNATIONAL SEARCH REPORT

Intern: al Application No
PCT/GB 98/02908

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 2 February 1999 | 11/02/1999 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Routledge, B |

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